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(54) Title: NON-ISOTOPIC NUCLEIC ACID LABELLING METHOD (57) Abstract A method for preparing a protein labelled nucleic acid probe t performing an extension reaction on a nucleic acid tem- plate in the presence of a nucleoside triphosphate chemically modified for covalent linkage, e.g. by having amino or thiol termi- nating a 3 to 40 atoms long linker arm, and covalently linking this to a reactive protein label. Also described are diagnostic kits, an assay method and nucleic acid probes. The invention may be used in diagnostic medicine, forensic, agricultural, veterinary and food sciences.		

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NON-ISOTOPIC NUCLEIC ACID LABELLING METHOD

The present invention relates to a novel method for non-isotopic labelling of nucleic acids, in particular to the construction of DNA probes labelled with enzymes. Current methods for the preparation of enzyme-labelled DNA probes fall into two types: those involving a covalent link between the enzyme and the probe, and those where the link is mediated via a specific binding pair such as avidin and biotin, or hapten and antibody. This latter method is well suited to longer double stranded probes since it allows the introduction of the enzyme in the form of an enzyme binding partner conjugate, after the hybridisation of the probe to the conjugate has occurred. This means that there is no need to find denaturing conditions that keep the two strands of the probe apart and which do not interfere with enzyme activity. The former covalent method has been used almost exclusively with single stranded, synthetic oligonucleotide probes, where problems of selective denaturation do not apply and the hybridisation conditions employed are generally milder. The above issues notwithstanding, covalent labelling has a number of advantages.

Firstly, there is no need for conjugate incubation and washing steps after hybridisation and the user can proceed with generation of the signal. Secondly, there is always the potential with the non-covalent approach for non-specific binding of the enzyme binding partner conjugate which can give rise to high backgrounds. The drawback of the covalent approach is that it is difficult to apply to long probes which are double-stranded and not prepared by chemical synthesis.

There is a need for a labelling method which can incorporate the advantages of covalent linkages and which can be applied to longer, non-synthetic probes generated, for example, by enzyme-mediated extension reactions.

Kleppe *et al* in J Mol. Biol. (1971), 56, 341-361 disclose a method for the amplification of a desired DNA sequence. The method involves denaturation of a DNA duplex to form single strands. The denaturation step is carried out in the presence of a sufficiently large

excess of two nucleic acid primers which upon cooling hybridise to regions adjacent to the desired DNA sequence.

Two structures are thus obtained each containing the full length of the template strand appropriately complexed with primer. DNA polymerase and a sufficient amount of each required deoxynucleoside triphosphate are added whereby two molecules of the original duplex are obtained. The above cycle of denaturation, primer annealing, and extension are repeated until the appropriate number of copies of the desired DNA sequence is obtained. It is indicated that adjustment of the primer concentration may be required. The above method is now referred to as the polymerase chain reaction (PCR), as described for example in European Patent Application, EP 201184.

It is becoming increasingly common to define DNA probes by reference to the DNA source (e.g. human genomic DNA) and the sequence of pairs of PCR primers, such that the scientist can readily perform the PCR reaction to obtain the desired probe.

Conventionally, such probes would be labelled by use of radiolabelled nucleoside triphosphates.

Alternatively, biotin or hapten labelled nucleotides could be employed and the probe detected after hybridisation as described above..

An important aspect of the present invention is a method for the preparation of probes by an extension reaction, such as the PCR reaction, which are labelled with protein labels, for example, an enzyme, via a covalent linkage. A preferred aspect of the invention therefore benefits from the advantages of the PCR method for defining and preparing DNA probes with the advantages of a covalent linkage between the label and the probe.

The method of the present invention is applicable to molecular biology research and to all areas of diagnostic medicine and other diagnostic sciences, for example forensic, agricultural, veterinary or food sciences where it is desirable to prepare probes for the detection or measurement of specific nucleic acid sequences. In particular it is applicable to the detection of infectious microorganisms and to the

detection of point mutations, gene deletions and rearrangements which give rise to various inherited diseases and predispositions.

According to the first aspect of the invention, there is provided a method for the preparation of a protein labelled nucleic acid probe which method comprises performing an extension reaction on a nucleic acid template in the presence of one or more nucleoside triphosphates chemically modified for covalent linkage to a protein label, and covalently linking the resulting extended nucleic acid product with a reactive protein label.

The term "nucleoside" as used in this specification and in the claims includes deoxynucleosides.

By extension on a nucleic acid template is meant any polymerase mediated extension of reaction whereby nucleotides can be incorporated into the growing nucleic acid strand. It includes known methods such as "primer extension" or "random hexamer labelling" (Feinberg A.P. and Vogelstein, Anal. Biochem 1983, 132,6 and Anal. Biochem. 1984, 137,266) and "nick translation" (Rigby P.W.J. et al., J. Mol. Biol, 1977, 113,237). It also includes RNA labelling systems such as those involving SP6 and T7 promoters (Melton D.A. et al., Nucl. Acid Res., 1984, 12, 7035). The extension reaction preferably includes terminally extending an oligonucleotide using one or more nucleoside triphosphates, which are chemically modified for covalent linkage to a protein label, in the presence of a suitable enzyme such as a terminal transferase. In this latter case it will be appreciated that the template can be a single strand of nucleic acids which is itself extended.

In a preferred aspect of the method of the invention the extension reaction is an amplification reaction ie. a reaction that can generate more than one copy of the sequence which is amplified.

The reactive protein label is preferably an enzyme, luminescent protein or a specific binding partner. Most usefully, the protein is an enzyme. Preferred enzymes are capable of generating a detectable signal, for example peroxidases such as alkaline phosphatase, horseradish peroxidase, beta-galactosidase, xanthine oxidase, and

firefly or bacterial luciferase which have been chemically modified to make them reactive to a nucleophile such as an amino or thiol group. Also, for those methods of the invention that do not provide single stranded probe, an enzyme that is stable to the commonly used methods for denaturing DNA, for example boiling, are preferred. Where protein labelled nucleic acid is intended to be hybridised with a target at high temperatures, e.g. 40-80°C, it is preferred that the enzyme is heat stable, for example stable in the range 40-80°C for a useful period of time, e.g. more than 30 minutes. These may be obtained, for example, from a thermophilic organism, or by engineering a less stable enzyme to increase heat stability. Frequently the desired stringency in hybridisations is achieved by denaturants such as formamide or urea. Enzymes stable to such conditions in addition or instead of heat stability are likewise preferred. Preferred methods of making reactive protein labels are described in our European Patent Application 431882. A particularly convenient and preferred enzyme is alkaline phosphatase. Luminescent proteins include the bioluminescent proteins such as obelin and aquorin, and the fluorescent proteins such as the phycobilliproteins. Specific binding partners include avidin, streptavidin and specific monoclonal or polyclonal antibodies.

The amplification reaction is conveniently a PCR reaction as described above. Because of the need to denature double-stranded probes and because of the lability of most signal proteins to such denaturing conditions it is preferable that the PCR product be substantially single stranded, that is it contains sufficient single stranded material to give a probe of the required sensitivity without denaturation. Such substantially single stranded PCR products can be generated for example by performing a PCR reaction with one primer in excess over the other, as described for example by Gyllensten U. B. et al., P.N.A.S., 1988, 85, 7652. For example a standard 100 ul PCR reaction employing 100 pmole of a first primer and 2 pmole of a second primer in combination with a target nucleotide sequence will produce an excess of the strand generated by the first primer. An alternative approach is to perform the PCR with one of the primers phosphorylated at

the 5' end. After the PCR reaction the product is then treated with for example lambda exonuclease which digests the strand containing the 5' phosphate to give a single stranded product. Such an approach is described by Higuchi R. G. et al., Nucl. Acid. Res., 1989, 17, 5865.

Alternatively other nucleic acid amplification methods based on polymerase-mediated extension reactions may be employed for example the transcription based system of Siska Diagnostics Inc., described in PCT patent application WO 88/10315.

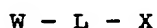
Most conveniently the amplification reaction employed in the method of the invention is the PCR reaction or one of its variants.

The amplification reaction may also be an enzyme mediated terminal extension reaction, such as any enzyme mediated extension reaction at one (or both) of the two termini of an oligonucleotide. In particular it includes forming a "tail" at the 3' end of the oligonucleotide in an extension reaction mediated by a terminal transferase enzyme. Accordingly, this aspect of the invention specifically includes a method for the preparation of a protein labelled nucleic acid probe which method comprises performing an extension reaction mediated by a terminal transferase enzyme, preferably on the 3' end of an oligonucleotide in the presence of one or more nucleoside triphosphates chemically modified for covalent linkage to a protein label, and covalently linking the resulting extended nucleic acid product with a reactive protein label. In this aspect of the invention the extension reaction preferably uses the 3' end of the previously formed oligonucleotide as an originating template and the terminal transferase catalyses terminal linking of any available nucleoside triphosphate. This generates an oligonucleotide of desired sequence with 3' "tail" of one or more randomly positioned bases including, according to the invention, at least one nucleotide which is adapted for covalent linkage to a protein label. The length and base composition of the "tail" can be varied by appropriate adjustment of the reaction conditions, in particular by varying the concentration of the reagents, especially terminal transferase and nucleoside triphosphate(s), and the reaction time. The length of the "tail" can be assessed by, for

example, gel electrophoresis. Typically the length of the tail will be long enough to ensure the incorporation of an adequate number of sites for covalent linking to a protein, but not so long that the "tail" interferes with hybridization of the probe section of the oligonucleotide. The "tail" can be as short as one base but can be substantially longer. The use of dideoxynucleoside triphosphate (chemically modified for covalent linking to a protein) as the nucleoside triphosphate(s) will prevent the extension reaction adding further bases (after the dideoxy base) because the dideoxy base residue lacks a 3'-hydroxyl group to act as the site for linking further bases to the nucleotide chain. This provides a convenient way of controlling the terminal transferase mediated extension reaction so as to extend the pre-formed oligonucleotide by a single base only.

This aspect of the invention is particularly valuable since it enables the preparation of oligonucleotide probes which are covalently linked or linkable to proteins which are, for example, an extension of an oligonucleotide which is known to be suitable as a probe for a particular application and in which the oligonucleotide probe in itself chemically unmodified as the modification is in the "tail".

The nucleoside triphosphate chemically modified for covalent linkage is preferably of the formula:

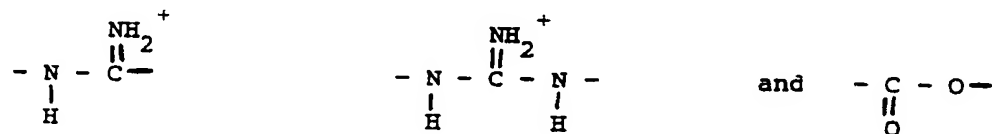


wherein W is a nucleoside or dideoxynucleoside triphosphate, L is a linking group and X is an optionally protected chemically reactive group.

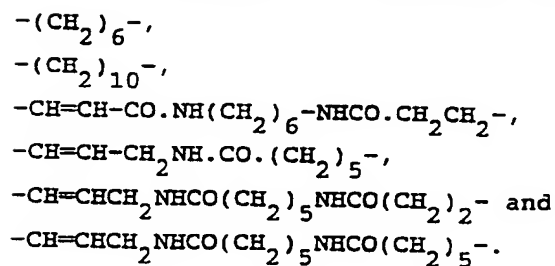
As examples of nucleoside triphosphates there may be mentioned ATP, GTP, CTP, TTP and UTP, and analogues thereof. It will be understood that where the extension reaction is a DNA extension reaction the group represented by W will be deoxynucleoside triphosphates.

The nature of the linker group L is designed to space the group X a sufficient distance from W such that (a) the compound of formula W - L - X is able to be recognised by a polymerase and

incorporated in a growing nucleic acid chain during an amplification reaction, and (b) the compound of formula W - L - X, once incorporated in a nucleic acid chain, is preferably able to base pair to a complementary base, that is the hybridisation ability of the probe produced by the amplification reaction should preferably not be significantly affected. The linker group L is preferably a divalent organic linker group of length 3 to 40 atoms, more preferably 6 to 30 atoms and especially from 10 to 25 atoms. L is preferably an optionally substituted and optionally interrupted hydrocarbon chain. The optional interruptions are preferably selected from -CH=CH-, -CONH-, -S-, -O-, -SO₂-, ureido, phenylene, cyclohexylene and groups of the formula:



Examples of preferred groups represented by L include:



For the avoidance of doubt 1,2-, 1,3- and 1,4-phenylene groups are respectively regarded as 2, 3 and 4 carbon atoms long.

The optional interruptions are incorporated in order to facilitate the synthesis of the chain and/or to provide more hydrophilic elements to maintain the linear structure of the chain and prevent it folding in on itself.

The site of attachment of the linker group L on the nucleoside triphosphate is most conveniently on the base moiety. In order not to interfere with hybridisation it is preferable that L should be joined to those atoms that are believed to be exposed in the major groove of the

DNA double helix that is C5, C6 or the amino group on C4 of cytosine; C5, C6 or the oxygen attached to C4 of uridine; C5 methyl, C6 or the oxygen attached to C4 of thymine; N7, C8 or the amino group on C6 of adenine; N7, C8 or the oxygen attached to C6 of guanine.

When X is a chemically reactive group which is not protected it is preferably a nucleophilic group or a group which is capable of being displaced by nucleophilic substitution to form a covalent bond between L and a nucleophile. As examples of such nucleophilic groups there may be mentioned groups of formula -NH_2 , -SH , imidazolyl, -NRH (wherein R is optionally substituted alkyl, such as C_{1-4} -alkyl), -COOH , and anions of such groups.

When X is a protected chemically reactive group it is preferably a protected nucleophilic group, especially a protected amino group or more preferably a protected thiol group; protecting groups for amino and thiol groups will be apparent to the organic chemist, and generally comprise a group which is stable under the normal aqueous conditions used for extension reaction, and yet can be removed chemically when desired.

Thiol and amino groups may be protected by any of the methods described in "Protective Groups in Organic Synthesis" by T.W.Green, Wiley Interscience. It is preferred that X is a protected thiol since we have found that there is a risk of unprotected thiols being oxidised by air or dimerising during the method of the invention. Thus modified nucleosides in which X is a protected thiol generally are more stable than the corresponding unprotected thiol, and this provides a particularly useful aspect of the invention. Examples of thiol protecting groups are given in Chapters 6 and 8 of T.W.Green's book, all of which are incorporated herein by reference thereto. Preferred protected thiols represented by X are those which can be converted to a free thiol under conditions which do not degrade DNA or RNA, for example thio ethers, thio esters, disulphides and sulphenyl derivatives.

As examples of preferred thio ethers there may be mentioned optionally substituted S-diphenylmethyl and S-triphenylmethyl thioethers such as S-diphenylmethyl, S-bis(4-methoxyphenyl)methyl,

S-5-dibenzosuberyl, S-triphenylmethyl, S-diphenyl-4-pyridylmethyl, S-phenyl and S-2,4-dinitrophenyl methyl thioethers; S-t-butyl and S-1-adamantyl thio ethers; hemithio, dithio and aminothio acetals; substituted S-methyl groups such as S-methoxymethyl, S-isobutoxymethyl, S-2-tetrahydropyranyl, S-benzylthiomethyl, S-acetamidomethyl, S-benzamidomethyl, S-acetyl-, S-carboxy- and S-cyanomethyl thio ethers; substituted S-ethyl groups such as S-2-nitro-1-phenylethyl; S-2,2-bis(carboethoxy)ethyl; and S-1-m-nitrophenyl-2-benzoylethyl.

Preferred thio esters represented by X include S-acetyl; S-benzoyl; S-thiobenzoyl; thiocarbonates such as S-2,2,2-trichloroethoxycarbonyl; S-t-butoxycarbonyl; S-benzyloxycarbonyl and S-p-methoxybenzyloxycarbonyl; and thiocarbamates such as S-(N-ethylcarbamate) and S-(N-methoxybenzyloxycarbamate).

Preferred disulphides represented by X include -S-S-ethyl, -S-S-t.butyl, and especially -S-S-aryl. The -S-S-aryl groups, which form a further aspect of the invention, especially those which can act both as a protecting group and a reactive group are particularly preferred due to their stability during the extension reaction and their ability to react with nucleophilic groups in reactive protein labels to form a covalent bond with the protein. Preferred -S-S-aryl groups are -S-S-(optionally substituted phenyl) groups such as -S-S-phenyl and -S-S-(3-carboxy-4-nitrophenyl); -S-S-pyridyl groups, especially -S-S-(2- or 4-pyridyl). The -S-S-pyridyl and -S-S-(3-carboxy-4-nitrophenyl) are particularly preferred as cleavage of the -S-S-disulphide bridge produces a compound which is detectable by UV spectroscopy, thereby allowing successful cleavage to be determined.

Preferred sulphenyl derivatives represented by X include S-sulphonate and S-sulphenyl thio carbonate.

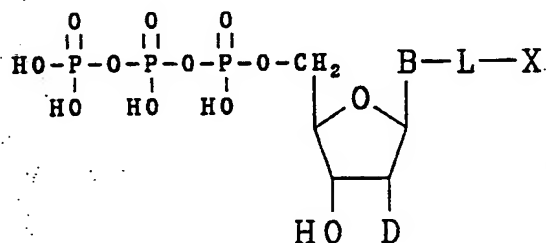
Examples of protected amino groups represented by X will be apparent to organic chemists, examples are fully described in Chapter 7 of the above mentioned book by T.W.Green and are incorporated herein by reference thereto. Suitable protected amino groups include carbamates, amides, N-benzyl derivatives, imine derivatives, enamines and N-hetero

atom derivatives such as N-sulphonyl and N-sulphenyl groups. However, generally speaking amino groups are more stable than thiol groups and protection in many cases is not necessary.

In the method of the present invention the resulting extended nucleic acid product may optionally be further modified, for example by addition of a thiol group (e.g. by reaction of an amino group with 2-iminothiolane) before covalently linking with the reactive protein label.

It is preferred that X is an optionally protected sulphur group because it is found that thiols are more nucleophilic than amines and covalent linkage with reactive protein is thereby facilitated. The protected thiol groups can be deprotected (i.e. converted to an -SH group) using appropriate deprotection conditions known to organic chemists such as those described in the above mentioned book by T.W.Green. For example, disulphides can be cleaved by mild aqueous reduction to give free thiols.

A particularly preferred modified nucleoside of the general formula W - L - X for use in the invention is shown in the free acid form by Formula (1):



(1)

wherein D is OH or preferably H; B is a divalent uracil, thymine, cytosine, adenine or guanine base; L is a linker group as hereinbefore described; and X is -SH, -NHR or -S-P¹ wherein R is H or C₁₋₄-alkyl, and P¹ is a protecting group. As will be apparent, after the modified nucleoside has been incorporated into a strand of nucleic acids the protecting group P¹, when present, should be removed to yield a free thiol group capable of covalently bonding to a reactive protein.

The reactive protein derivative is a protein label, as described above, which has in general been rendered reactive such that it is able to form a covalent bond with the group X on the amplification product.

Suitable pairs of reactive groups are known in the literature, for example in "Practice and theory of enzyme immunoassays" by P.Tijssen, Elsevier, 1985 and in the Catalog of the Pierce Chemical Company.

The protein may be rendered reactive by derivatising its amino acid side chains, such as its amino groups, imidazole groups, guanidine groups, thiol groups (whether native or generated from disulphides by reductive cleavage) or carboxyl groups. Glycoproteins may also be derivatised at the carbohydrate portion. The most convenient group to convert to a reactive group is the amino group.

The number of groups in the protein which are rendered reactive should preferably be kept low so as to minimise the chance of interfering with the protein's activity and to minimise the possibility of non-specific binding of the conjugate formed by the derivative. Therefore in general the number of reactive groups should be less than 5 moles per mole of protein, more preferably from 1 to 3 moles per mole of protein, most preferably 1 or 2 moles per mole of protein.

The side chains of the protein can be derivatised using a haloacetyl, haloacetamidyl, maleimido, sulphonyl halide, -N=C=S, -C=NH₂.O-(C₁₋₄-alkyl), an -S-S-pyrid-2-yl or an -S-S-pyrid-4-yl reactive group. Maleimido is particularly useful. Such groups may be attached to the protein by an optionally substituted saturated or unsaturated hydrocarbon chain containing for example up to 12, up to 10, up to 8, up

to 6, up to 4 and conveniently up to 8 carbon atoms. Examples of optional substituents include hydroxyl groups. It will be appreciated that the chosen substituents should not interfere with the linking chemistry. Several reagents able to introduce reactive groups into proteins have been described in the literature. For convenience active ester reagents capable of reacting with protein amino groups are preferred, such as optionally substituted N-hydroxysuccinimide esters. A particularly convenient reagent is the succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate (SMCC) as described and prepared by Yoshitake *et al.*, European J. Biochem., 1979, 101, 395-399. This reagent gives a protein derivative containing a reactive maleimido group.

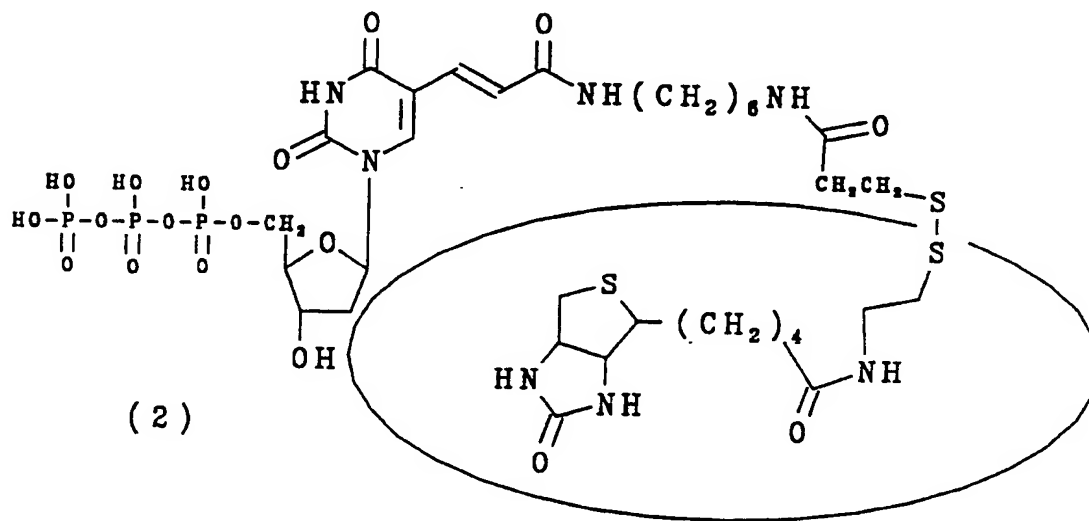
In a preferred aspect of the invention, the reactive protein derivative is in a stable, pre-activated form as described in our European Patent Application No.431882.

The term "stable" means that it can withstand commonly employed storage temperatures of for example -20°C, 4°C or ambient temperatures for useful periods of time, for example more than one month, preferably more than three months, most preferably more than six months, either in solution or in the lyophilised state. Such derivatives are usefully employed in a labelling kit using the method of the invention.

The conditions selected for covalently bonding the extended nucleic acid product with the reactive protein depend upon the nature of the extended nucleic acid product, e.g. the nucleophilicity of any groups represented by X, and the reactivity of the protein. In general the covalent binding will occur in an aqueous buffer of neutral or moderately alkaline pH at temperatures between 2 and 40°C. Optimum conditions for the covalent binding may be determined in individual cases by experiment. After the extended nucleic acid product and reactive protein have been covalently bonded there may be significant quantities of unreacted protein present together with other reactants or biproducts present. In order to reduce the non-specific binding properties of the probe mixture produced, it may in some circumstances

be considered desirable to remove this unreacted protein and other impurities. Accordingly the method provides for an optional purification step after the conjugation step. This may be achieved by any convenient means for example chromatography on a gel filtration, ion-exchange, hydrophobic, reverse phase or affinity column or ultrafiltration using a membrane of suitable pore size. It is preferred that any purification is brought about using a gel filtration column, conveniently a small disposable gel filtration column.

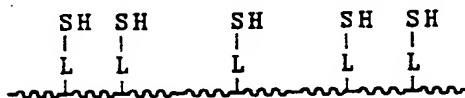
A particularly preferred group of the formula W - L - X is shown in its free acid form by Formula (2) wherein the encircled portion is a protecting group:



The compound of Formula (2) is hereinafter referred to as dUTP-21-SS-biotin.

The PCR reaction is preferably asymmetric, or alternatively the exonuclease version of the PCR as hereinbefore described. After the PCR reaction is complete, and in the case of the exonuclease version of the PCR reaction, the exonuclease step has been performed, any

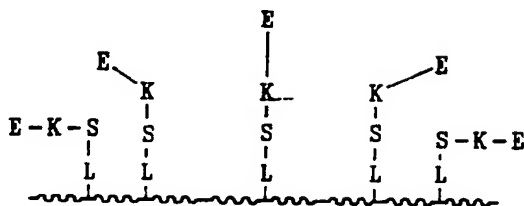
protecting groups in the product may be removed. For example when W-L-X is of Formula (2) the product is treated with a mild reducing agent, for example dithiothreitol, which cleaves the disulphide group thereby excising the encircled protecting group, and the mixture subjected to gel filtration, for example on Sephadex G25, which preferably gives a single stranded thiolated PCR product of the formula:



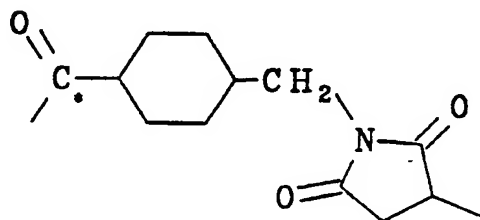
wherein the wavy line is a strand of DNA, L is a linker group, and SH is a thiol group. This product is then reacted with a reactive protein label.

The reaction of extended nucleic acid product and reactive protein label, for example a protein label to which maleimido groups have been attached, is allowed to proceed at approximately neutral or moderately alkaline pH for typically at least one hour after which the covalently bonded product may be purified by for example one of the methods described above.

The resulting covalently bonded protein and extended nucleic acid is represented by the formula:



where E is a protein, L is a linker group, S is sulphur, and K is, for example:



in which the asterisked carbonyl is attached to the protein E.

In a further aspect of the invention there is provided a protein labelled nucleic acid probe prepared by the method of the invention.

In a still further aspect of the invention there is provided an assay method for detecting the presence, absence or amount of a target nucleic acid in a sample containing other nucleic acid sequences which are not sought to be detected, which method comprises contacting the protein labelled nucleic acid of the present invention with the sample under hybridisation conditions, optionally removing unhybridised

probe, and then detecting the presence or absence of hybridisation by means of the protein label.

The target nucleic acid may be from any source and is preferably of biological origin or an amplification product thereof. It is convenient to bind the target to a solid support such as a membrane, filter, bead or microtitre plate well, either directly or via other binding moieties according to the nature of the assay. A useful application is the detection of target nucleic acids bound to nylon or nitrocellulose filters arising from Southern blot, Northern blot, slot blot, dot blot, plaque lift or colony lift experiments. A further use for the probe of the present invention is for the detection of nucleic acid sequences in situ, i.e. in tissues.

The expression "hybridisation" as used herein includes hybridisation between a polynucleotide probe and a desired target sequence but excludes hybridisation between a polynucleotide probe and non-desired nucleotide sequences. Suitable conditions for hybridisation are well known to the scientist of ordinary skill, see for example "Nucleic Acid Hybridisation", B. D. James and S. J. Higgins (Eds), IRL Press, Oxford, 1985.

In a further aspect of the invention there is provided a kit for the preparation of protein labelled nucleic acid probes by the method described herein. The kit of the invention comprises:

- (i) a nucleoside triphosphate chemically modified for covalent linkage to a protein label; and
- (ii) a reactive protein label or a protein label with an agent capable of converting it to a reactive protein label.

The preferred reactive protein label and chemically modified nucleoside triphosphate are as described above.

The kit preferably also contains one or more component selected from the following list: components for effecting the extension or amplification reaction, for example a polymerase or terminal transferase; a buffer for the amplification or extension reaction; the triphosphate of adenosine, guanosine, cytidine or thymidine; an exonuclease (for kits using the exonuclease method of

generating single stranded PCR probes); components to effect the subsequent conjugation reaction, for example an agent for the deprotection of the chemically reactive group (where appropriate); a column for the removal of excess deprotection agent; reaction and column buffers; a column or other devices for the means of purification of the final conjugate; an agent for the further chemical modification on the resultant extended nucleic acid product e.g. 2-iminothiolane; and a protocol for carrying out the procedure according to the present invention.

The invention is illustrated by, but not limited to, the following examples:

Abbreviations

The following abbreviations and definitions apply to the Examples:

Buffer A	: 0.1M triethanolamine-HCl pH 7.4, 1 mM $MgCl_2$, 1 mM $ZnSO_4$.
Buffer B	: 67 mM glycine - NaOH pH 9.4, 2.5 mM $MgCl_2$.
Buffer C	: 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin.
Buffer H	: 0.1% BSA, 0.1% PVP, 0.1% Ficoll 400 1% SDS, 5 x SSC
Wash solution 1:	1 x SSC, 0.5% SDS
Wash solution 2:	0.25 x SSC, 0.5% SDS
dUTP-21-SS-biotin;	Purchased from Clontech, see Formula (2)
SMCC	succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate Purchased from Pierce
Tris	tris(hydroxymethyl)aminomethane
BSA	bovine serum albumin
PBS	0.13M NaCl, 5.4 mM Na_2HPO_4 , 1.6 mM KH_2PO_4 buffer pH 7.3
SSC	20x SSC is 3M NaCl, 0.3M trisodium citrate
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulphate
DMF	dimethyl formamide
DTT	dithiothreitol

METHODS

Preparation of the reactive protein maleimido alkaline phosphatase ("mal AP")

A sample of alkaline phosphatase (hereinafter "AP") was made reactive towards -SH groups by reaction with SMCC as follows:

To a solution of alkaline phosphatase (Boehringer, 10 mg/ml, 0.2 ml) was added 0.1M triethanolamine HCl, 1 mM MgCl_2 , 1 mM ZnSO_4 , pH 7.4 (0.6 ml), followed by 12 microlitres of a freshly prepared solution of SMCC (Pierce) in dry DMF (6.7 mg/ml) and the reaction mixture incubated at 25°C for 30 min. The product was then purified by passage through a NAP 25 desalting column (Pharmacia), primed with BSA (Boehringer molecular biology grade) and equilibrated in PBS or, to prepare a stable lyophilised preparation, in 0.010 M sodium phosphate buffer pH 7.4 containing 10 g/l D-lactose. The product was collected in 1.6 ml and a portion taken for analysis.

Protein concentration was assessed by OD at 280 nm (using an extinction coefficient of 0.89 for a 1 mg/ml solution) whilst the maleimido concentration was assessed as follows: 0.15 ml of sample was reacted with 10 microlitres of 1 mM mercaptoethanol for 30 min. at 37°C, alongside a control with 0.15 ml of buffer alone. The reactions were then diluted with 1.2 ml of PBS, zeroed at 412 nm in a spectrophotometer, and 25 microlitres of 1 mM 5,5', dithiobis(2-nitro-benzoic acid) added. Remaining thiol concentrations were thereby measured using an extinction coefficient of 14150, the difference between sample and control enabling the maleimido concentration and hence the degree of substitution to be calculated. This value was found to be between 1.4 and 1.6 moles maleimido per mole of protein. For the preparation of the stable, lyophilised preparation, the derivatised enzyme was then aliquoted into 3 nmole portions and lyophilised.

Preparation of oligonucleotides

The following oligonucleotides were synthesised on an Applied Biosystems automated DNA synthesiser using protocols recommended by the manufacturer:

Primer 1: GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG
Primer 2: CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG
Primer 3: GAC TTC ACT TCT AAT GAT GAT TAT GGG AGA
Primer 4: CTC TTC TAG TTG GCA TGC TTT GAT GAC GCT

Primer 2 was also synthesised with a 5' phosphate group prepared using the phosphoramidite "Phosphate ON" (Cruachem) using the manufacturer's protocol.

After deprotection and evaporation, each sample was redissolved in 1.0 ml of water.

Amplification of Target DNA samples by PCR

Human genomic DNA was amplified as follows: to an Eppendorf microfuge tube was added the dNTP mix (4 microlitres, 1 mM each), primers 1 and 2 (for amplification of alpha-1 anti-trypsin exon 3, 100 pmoles of each primer) or primers 3 and 4 (for amplification of cystic fibrosis gene exon 10, 100 pmoles of each primer), Buffer C (10 microlitres), human genomic DNA (30 ng in 6 microlitres, water to make up to 99 microlitres and 2.5 U of Taq polymerase (Perkin Elmer Cetus, 1 microlitre). The contents of each tube were mixed, centrifuged and covered with light mineral oil (Sigma) and subjected to thermal cycling on a Perkin Elmer Cetus thermal cycler programmed as follows:

15 cycles of: 94°C, 2 minutes; 60°C, 2 minutes; 72°C,
30 seconds

followed by

35 cycles of: 94°C, 45 seconds; 64°C, 45 seconds; 72°C,
90 seconds.

Preparation of Slot Blots

For slot blot preparation, a yield of 1 microgramme of DNA/100 microlitres symmetrical PCR product target was assumed and used to calculate dilutions. Dilutions in water were performed to give the following amounts in 1 microlitre: 1000, 100, 10, 1 pg. Denaturation buffer was prepared as follows:

to 1.48 ml of water was added 0.17 ml 1M Tris. HCl pH 7.5, 0.30 ml 2M NaOH and 1.0 ml of 20x SSC. PCR product target (1 microlitre) was added to denaturation buffer (75 microlitres) and the samples incubated in a boiling water bath for 10 minutes, transferred to an ice bath and neutralised with 25 microlitres of 1M Tris. HCl. Slot blots were prepared using Hy-bond nylon membrane (pre-soaked in 1x SSC), washing the slots first with 1x SSC (0.4 ml), applying the sample of PCR product target and further washing with 1x SSC (0.4 ml). The membranes were air-dried on filter paper for 20 minutes, wrapped with Saran-wrap, UV-irradiated (to cross link the PCR product target to the membrane) for 3 minutes and further air-dried overnight.

Hybridisation procedure

Slot blots were incubated in Buffer H for 2 hours at 50°C in a shaking water bath. The membranes were then sealed in hybridisation bags with 5 ml of fresh Buffer H containing nucleic acid probe and incubated for 20 minutes at 50°C. After hybridisation, the membranes were washed twice in wash solution 1 for 5 minutes at 50°C, twice in wash solution 2 for 5 minutes at 50°C and once in 1 x SSC for 5 minutes at room temperature to remove unhybridised probe. The membranes were placed on a plastic tray and sprayed with Lumi-Phos (Lumigen Inc.), and then sealed between acetate sheets, incubated at 37°C for 1 hour and exposed to X-ray film (Fuji). An initial activation period of 1 hour at 37°C was allowed for development of the signal.

Example 1

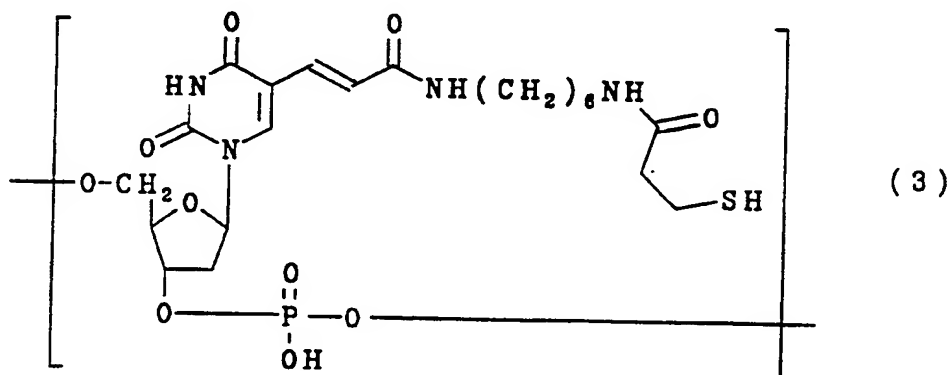
Preparation and use of a labelled cystic fibrosis (CF) probe using an asymmetric PCR version of the method

A sample of human genomic DNA was amplified as described under "preparation of target" but with the following modifications:

Primer 3 was used at 1 pmole, primer 4 at 100 pmole. The concentration of dTTP was 20 micromolar. The reaction included dUTP-21-SS-biotin at a concentration of 20 micromolar. 3 PCR reactions were performed and the products pooled.

After the PCR reaction dUTP- 21-SS-biotin (which is a nucleoside triphosphate chemically modified for covalent linkage to a protein label) had been incorporated into the PCR product to give a DNA strand containing a disulphide linkage. The disulphide linkage was cleaved by incubating 25 microlitres of the PCR product for 10 minutes at room temperature with DTT to give a solution of -SH labelled DNA.

The solution was then passed through an NAP-25 desalting column to remove excess DTT, the -SH labelled DNA was eluted in 1.6 ml of Buffer A to give a DNA strand containing one or more units of Formula (3) and collected in a tube containing 3 nmoles of freeze-dried mal AP.



The -SH labelled DNA was reacted with mal AP by incubation therewith overnight at 4°C to form a covalent bond between AP and the sulphur atom of the -SH by displacement of maleic leaving groups present in mal AP. The DNA labelled via the sulphur group to alkaline phosphatase is hereinafter referred to as the AP labelled CF probe.

The AP labelled CF probe was separated from free alkaline phosphatase and other impurities on a Sephacryl S-200 gel column (12 ml) equilibrated with PBS. Fractions were analysed by UV spectrometry and the first fractions, thereby shown to contain AP labelled CF probe, were

pooled. The AP labelled CF probe was made up to a final volume of 2.2 ml including 0.1% BSA and 0.2% sodium azide.

CF slot blots, prepared as described above under "Preparation of Slot Blots" were each probed with 0.5 ml of the above solution of AP labelled CF probe (1 nM) using the hybridisation procedure described above, and after the washes to remove unhybridised probe the membranes were treated with Lumi-Phos as described, and exposed to X-ray film for 5 minutes. A positive signal was observed with the 10 pg target CF DNA band. No signal was observed on a negative control (alpha 1 AT) slot which did not contain a CF DNA band.

Example 2

Preparation and use of an alpha-1 antitrypsin exon 3 probe (alpha 1 AT) using the exonuclease version of Example 1.

An extension reaction was performed on a sample of human genomic DNA using the method described under "preparation of target" but with the following modifications:

Primer 1 and 5' phosphoryl primer 2 were used (100 pmole each each) The concentration of dTTP was 20 micromolar. The reaction included dUTP-21-SS-biotin at a concentration of 20 micromolar. 2 PCR reactions were performed.

DNA from 170 microlitres PCR product was extracted once with phenol/chloroform (1:1, 170 microlitres), once with chloroform/isoamyl alcohol (24:1) and precipitated with ethanol (3 volumes). The DNA was redissolved in 50 microlitres Buffer B and treated with 4 units of lambda exonuclease (obtained from BRL), for 15 minutes at 37°C to produce single stranded DNA in which at least one dUTP-21-SS-biotin unit had been incorporated. DTT was added to a final concentration of 10 mM and the solution was incubated at room temperature for 10 minutes to cleave the disulphide bridge, thereby converting any dUTP-21-SS-biotin units in the DNA to a unit of Formula (2) having the free -SH group. The resultant solution was passed through a NAP-5 desalting column (Pharmacia). The solution was eluted in 0.6 ml PBS and collected in a tube containing 125 microlitres of freshly prepared mal AP (approximately 1.8 nmols/ml). The solution was mixed and incubated

overnight at 4°C to allow any -SH groups in the sample to form a covalent bond with alkaline phosphatase by nucleophilic displacement of maleic reactive groups. The resultant DNA labelled via a sulphur group to alkaline phosphatase is hereinafter referred to as the AP labelled alpha 1 AT probe.

The AP labelled alpha 1 AT probe was separated from free alkaline phosphatase and other impurities on a Sephacryl S- 200 gel column eluted with PBS, eluting with 1.5 ml PBS, the volume was reduced to 1 ml on a speedy-vac concentrator and this was used to probe one alpha 1 AT slot blot, using the hybridisation procedure described above and a probe concentration of 1 nM.

A 20 minute exposure to film gave a positive signal with 1 pg target alpha 1 AT DNA. No signal was observed on the negative control slots which did not contain target alpha 1 AT DNA.

Example 3

Preparation and use of a 33.6 oligonucleotide probe labelled by the terminal transferase method

(i) Preparation of 6-trifluoroacetamido hexanoic acid

To a suspension of 6-aminohexanoic (13.2g, 100mmoles) in dry DMF (50ml) containing triethylamine (10.12g, 13.94ml, 100mmoles) at 0°C, was added trifluoroacetic anhydride (14.2ml, 100mmoles) dropwise with stirring. A clear yellow solution resulted which was allowed to warm to room temperature and stirring was maintained overnight. Water (20ml) was added, and the solution was evaporated to an oil which was partitioned between ethyl acetate (200ml) and water. The layers were separated and the organic phase was washed with saturated sodium bicarbonate solution (2 x 200ml) and water (2 x 200ml). The organic phase was dried over anhydrous sodium sulphate, filtered and evaporated to a beige solid (11g) which was used without further purification.

(ii) Purification of 6-trifluoroacetamide-hexanoic acid succinimide

To a solution of 6-trifluoroacetamide hexanoic acid (2.3g, 10mmoles) in dry DMF (10ml) were added dicyclohexyl carbodiimide (2.27g, 11mmoles) and N-hydroxysuccinimide (1.15g, 10mmoles). The solution was stored at room temperature overnight, by which time a dense white precipitate had formed. The suspension was stored at 4°C for 4 hours and then filtered. The filtrate was used directly without further purification.

(iii) Preparation of 5-allylamino-2'-deoxyuridine

5-Trifluoroacetamide allyl-2'-deoxyuridine (prepared analogously to (i)) was dissolved in concentrated ammonia solution (20ml, BDH) and heated at 60°C for 2 hours when TLC in $\text{CHCl}_3/\text{MeOH}$ (4/1) showed there to be no starting material left. The solution was evaporated to an oil which was co-evaporated several times with methanol and then redissolved in water (100ml). This was washed with diethyl ether and the aqueous layer was evaporated to an oil which was used in the next step without further purification.

(iv) Preparation of 5-(1-propenyl-3-(amino hexanoyl-6-trifluoroacetamide)-2'-deoxyuridine

To a solution of 5-allylamino-2'-deoxyuridine (1g, 3.5mmole) and triethylamine (0.5ml) in dry DMF (20ml) was added 3.5ml of the DMF solution from step 2). The solution was kept at room temperature overnight, and was then evaporated to an oil which was redissolved in $\text{CHCl}_3/\text{MeOH}$ (4/1) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless glass (1g, 57%).

¹H NMR: 5 (DMSO d⁶): 11.35, 1H, s, N³-H; 9.35, 1H, m, $\overset{\text{H}}{\text{NCOCF}_3}$;
 8.0, 1H, s, H-6; 7.9, 1H, t, $\overset{\text{H}}{\text{N-CH}_2}$; 6.4, 1H, m, =C-H;
 6.1, 2H, m, CH= + H-1'; 5.2, 1H, d, 3'-OH; 5.05, 1H, t, 5'-OH;
 4.2, 1H, m, H-3'; 3.75-3.65, 3H, m, H-4' + $\overset{\text{H}}{\text{CH}_2\text{-NCOCF}_3}$;
 3.6, 2H, m, H-5'; 3.2, 2H, m, $\overset{\text{H}}{\text{CH}_2\text{-NCO}}$;
 2.2, 4H, m, H-2' + CH₂CO; 1.5-1.3, 6H, m, (CH₂)₃.

(v) Preparation of 5-(1-propenyl-3-(amidohexanoyl-6-trifluoroacetamide))-2'-deoxy-5'-O-p-toluenesulphonyl uridine

To a stirred solution of the product of step (iv) (0.45g, 0.92mmol) in dry pyridine (10ml) at 0°C was added p-toluenesulphonyl chloride (0.19g, 1mmol). Stirring was continued at 0°C for 1 hour and the solution was allowed to warm to room temperature gradually. Stirring was maintained overnight, and then pyridine was removed by rotary evaporation. The residue was co-evaporated several times with toluene, redissolved in CHCl₃/MeOH (9/1) and applied to a silica column. Elution with the same solvent gave the title compound as a white solid.

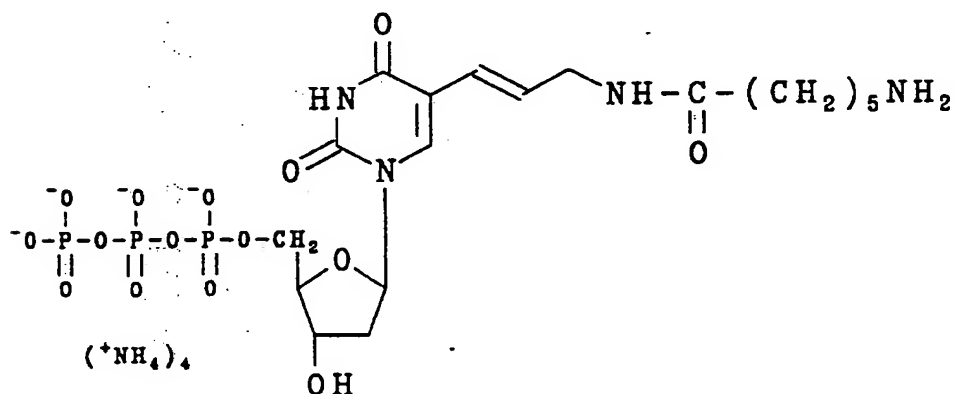
¹H NMR: (DMSO d⁶): 11.4, 1H, 2, N³-H; 9.3, 1H, m, $\overset{\text{H}}{\text{NCOCF}_3}$;
 7.9, 1H, t, $\overset{\text{H}}{\text{N-CH}_2}$; 7.8, 2H, d, 2xAr-H; 7.55, 1H, s, H₆;
 7.4, 2H, d, 2xAr-H; 6.5, 1H, m, H-C=; 6.2-6.1, 2H, m, H-1' + =C-H;
 4.3-4.15, 3H, m, H-4' + H-5'; 3.94, 1H, m, H-3';
 3.8, 2H, d, $\overset{\text{H}}{\text{CH}_2\text{-NCOCF}_3}$; 3.2, 2H, t, $\overset{\text{H}}{\text{CH}_2\text{-NCO}}$;
 2.4, 3H, Ar-CH₃; 2.25-2.05, 4H, m, H-2' + CH₂CO;
 1.6-1.2, m, (CH₂)₃

(vi) Preparation of 5-(1-propenyl-3-[amidohexanoyl-6-trifluoroacetamide])-2-deoxyuridine-5'-triphosphate

To a stirred solution of the product from step (v) (0.22g, 0.36mmol) in dry acetonitrile (0.3ml) was added tetra (tetrabutyl ammonium) triphosphate (prepared as described in ref.2) (0.66g, 0.54mmole) and the resulting slurry was stirred at room temperature for five days.

The mixture was diluted with water (5ml), applied to a column of Dowex 50x-8 resin (NH_4^+ form) and eluted with water. UV absorbing factors were pooled and lyophilized. The resulting solid was extracted into 2ml of acetonitrile/100mM ammonium bicarbonate/conc NH_3 (7:3:2) and the soluble portion was chromatographed on cellulose using the same solvent system. UV absorbing fractions were pooled, acetonitrile was reviewed by rotary evaporation and the remaining solution was stored frozen.

(vii) Preparation of 5-(1-propenyl-3-[aminohexanoyl-6-amino])-2'-deoxyuridine-5'-triphosphate (aminocaproyl dUTP)



To 10ml of the aqueous solution resulting from step (vi) above was added concentrated ammonia solution (20ml, sp.gr. 0.880) and the solution was kept at room temperature overnight. The solution was lyophilized and the resulting solid was redissolved in water to a concentration of 4mM.

(viii) Labelling of synthetic oligodeoxynucleotides with alkaline phosphatase

(a) Extension reaction

An oligomer of sequence: 5'-GTGGATAGGGTGGATAGGGTGG (4nmoles) was incubated in a buffer containing 50mM KCl, 10mM Tris.HCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 9.0 in the presence of 20nmole of aminocaproyl dUTP and 75 units of terminal deoxynucleotidyl transferase in a total volume of 170-microlitres. The solution was incubated at 37°C for 2 hours. 18 microlitres of 3M sodium acetate and 470 microlitres of ethanol were added to the tube. After thorough mixing the tube was incubated at -70°C for 15 minutes and then spun in an Eppendorf microfuge for 15 minutes. The supernatant was discarded and the pellets were redissolved in 100 microlitres of 0.3M sodium acetate and 300 microlitres of ethanol was added to each. The tubes were again incubated at -70°C and spun in a microfuge. The resulting pellet was redissolved in water to give a final volume of 100 microlitres which was used directly in the next step.

(b) Covalent linkage of product from step (a) to a reactive protein

The extended oligonucleotide from step (a) was reacted with iminothiolane to convert the terminal amino group of formula $-(CH_2)_5NH_2$ into a group of formula $-(CH_2)_5-NH-C(=NH_2^+)CH_2CH_2CH_2SH$, and subsequently reacted with activated alkaline phosphatase by use of the E-LINK oligonucleotide conjunction kit (Cambridge Research Biochemicals) according to the manufacturer's protocol to give 1.1ml of product.

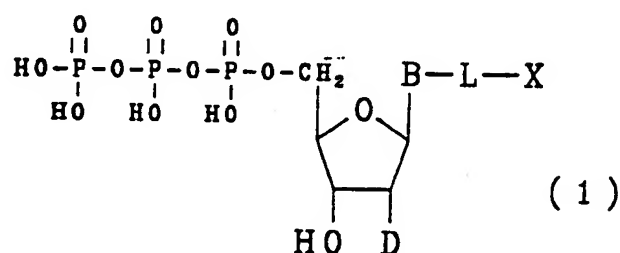
(ix) Testing of the protein labelled probe from step (b) on slot blots

Slot blots of full length probe MS1 (Cellmark Diagnostics) were prepared as described above with DNA loadings of 50, 10, 5, 1 and 0.1pg. These were hybridised with the labelled probe (50 microlitres) and detected with Lumiphos essentially as described above but with the pre-hybridisation time set at 1.5 hours and the film exposure time of 1 hour. A positive signal was observed with the 0.1pg band.

CLAIMS

1. A method for the preparation of a protein labelled nucleic acid probe which method comprises performing an extension reaction on a nucleic acid template in the presence of one or more nucleoside triphosphates chemically modified for covalent linkage to a protein label, and covalently linking the resulting extended nucleic acid product with a reactive protein label.
2. A method according to Claim 1 wherein the extension reaction is an amplification reaction.
3. A method according to Claim 1 wherein the extension reaction is a polymerase chain reaction.
4. A method according to Claim 1 wherein the extension reaction is mediated by a terminal transferase enzyme.
5. A method according to any one of the preceding claims wherein said nucleoside is of the formula W - L - X wherein W is a nucleoside or dideoxy nucleoside triphosphate, L is a linking group and X is an optionally protected reactive group.
6. A method according to Claim 5 wherein the linking group L is from 3 to 40 atoms long.
7. A method according to Claim 5 or Claim 6 wherein X is an optionally protected nucleophilic group.
8. A method according to Claim 5 or Claim 6 wherein X is thiol group, a protected thiol group or an amino group.
9. A method according to Claim 5 wherein X is a protected thiol group.

10. A method for the preparation of a protein labelled nucleic acid probe which method comprises performing an extension reaction on a nucleic acid template in the presence of one or more nucleoside which, in the free acid form, is of the Formula (1):



wherein D is H or OH; B is a divalent uracil, thymine, cytosine, adenine or guanine base; L is a linker group of length 3 to 40 atoms; and X is -SH, -NHR or -S-P¹ wherein R is H or C₁₋₄-alkyl and P¹ is a protecting group; removing the protecting group P¹ where necessary, and covalently linking the resultant extended nucleic acid product with a reactive protein label.

11. A method according to Claim 10 wherein X is of the formula -S-P¹ wherein P¹ is a protecting group.

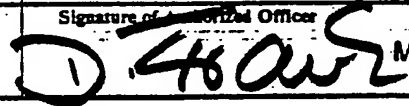
12. A method according to Claim 1 or Claim 10 wherein the reactive protein label is an enzyme.

13. A method according to Claim 1 or Claim 10 wherein the reactive protein label is an enzyme which has been chemically modified to make it reactive to a nucleophile.

14. A diagnostic kit comprising
- (i) a nucleoside triphosphate chemically modified for covalent linkage to a protein label; and
 - (ii) a reactive protein label or a protein label with an agent capable of converting it to a reactive protein label.
15. An assay method for detecting the presence, absence or amount of a target nucleic acid in a sample-containing other nucleic acid sequences which are not sought to be detected, which method comprises contacting a protein labelled nucleic acid (prepared by a method according to Claim 1) under hybridising conditions and then detecting the presence or absence or amount of target by means of the protein label.
16. A compound of the formula
- $$W - L - S - S - \text{aryl}$$
- wherein W is a nucleoside triphosphate; and
L is a linking group.
17. A compound according to Claim 16 wherein L is a linking group from 3 to 40 atoms long, and the aryl group is an optionally substituted phenyl group or a pyridyl group.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01112

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5 C 07 H 21/00 C 12 Q 1/68 C 07 H 19/04		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	C 07 H C 12 Q	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,8602929 (LIFE TECHNOLOGIES INC.) 22 May 1986, see page 3, line 18 - page 4, line 12; page 8, line 4 - page 9, line 17; claims 1,6,11,13 ---	1,10,15
X	US,A,4587044 (P.S. MILLER et al.) 6 May 1986, see the whole document, especially column 2, line 15 - column 3, line 60; column 10, lines 40-66; claims 41-44 ---	1,4,5,7,8,10,13
Y	---	2,3
Y	Biotechniques, volume 8, no. 6, 1990, Eaton Publishing Natick, MA(US), J.J. Lanzillo: "Preparation of digoxigenin-labeled probes by the polymerase chain reaction", pages 620-622, see the whole article --- -/-	2,3
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14-10-1991	11. 11. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 Mme Dagmar FRANK	

III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	US,A,4772691 (T.H. HERMAN) 20 September 1988, see abstract; column 8, lines 1-60; claims ---	16
Y	WO,A,8902932 (CETUS CORP.) 6 April 1989, see page 5, line 1 - page 9, line 18 ---	16
A	EP,A,0124221 (A.D.B. MALCOLM et al.) 7 November 1984, see page 23 ---	16
A	Biochemistry, volume 28, 11 July 189, American Chemical Society, (Washington, D.C., US), M.M. Hanna et al.: "Synthesis and characterization of 5-[(4-azidophenacyl)thio]uridine 5'-triphosphate, a cleavable photo-cross-linking nucleotide analogue", pages 5814-5820, see figures 1 and 2 -----	16

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9101112
SA 49266

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/11/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8602929	22-05-86	US-A- 4828979 EP-A- 0203959 JP-A- 61115094	09-05-89 10-12-86 02-06-86
US-A- 4587044	06-05-86	None	
US-A- 4772691	20-09-88	None	
WO-A- 8902932	06-04-89	EP-A- 0372002 JP-T- 2502695 US-A- 4962029	13-06-90 30-08-90 09-10-90
EP-A- 0124221	07-11-84	AU-B- 572505 AU-A- 2695184 WO-A- 8403520 JP-T- 60500651	12-05-88 28-09-84 13-09-84 09-05-85